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## Emergence of a colistin-resistant KPC-2-producing *Klebsiella pneumoniae* ST258 clone in Hungary

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**Abstract** Nine *Klebsiella pneumoniae* isolates showing non-susceptibility to carbapenems were collected from three centres in the north-eastern region of Hungary. The minimum inhibitory concentrations (MICs) of antibiotics were determined by Etest. The putative production of a carbapenemase was tested by the modified Hodge test. The presence of *bla*<sub>KPC</sub> genes was verified by polymerase chain reaction (PCR) and sequencing. Furthermore, molecular typing was performed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). All isolates showed extensively drug-resistant (XDR) phenotype,

and of these, eight isolates were highly resistant to colistin. The isolates carried *bla*<sub>KPC-2</sub>, *bla*<sub>SHV-12</sub>, *bla*<sub>TEM-1</sub> and *bla*<sub>SHV-11</sub>. PFGE analysis of the nine KPC-2-producing Hungarian ST258 *K. pneumoniae* isolates, two KPC-2-producing Norwegian ST258 isolates and 33 CTX-M-15-producing ST11 isolates revealed the existence of one genetic cluster at an 88% similarity level. The overall results of the PFGE clustering, MLST and the presence of SHV-11 in both ST11 and ST258 suggest that this is the first hyperepidemic clonal complex of multidrug-resistant *K. pneumoniae*, probably CC258/CC340, possibly undergoing worldwide spread.

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## Introduction

*Klebsiella pneumoniae* carbapenemases (KPC) belonging to the Ambler class A  $\beta$ -lactamases is able to hydrolyse virtually all  $\beta$ -lactam antibiotics, including carbapenems [1]. Since the first detection of KPC-producing *K. pneumoniae* in 1996 in the USA [2], these carbapenemases have been identified in many species of *Enterobacteriaceae*, but mostly in *K. pneumoniae*. During this decade, the prevalence of KPC-producing strains among *K. pneumoniae* has rapidly increased and the clonal dissemination of KPC-producing *K. pneumoniae* is currently being observed. Emergence and explosive dissemination of the KPC-producing *K. pneumoniae* ST258 international clone—which is a single-locus variant of the ST11 epidemic clone—has been reported recently from Israel, the USA [3], Norway, Sweden [4], Poland [5], Finland [6] and Italy [7]. This is quite alarming because the typically coupled resistance to several non- $\beta$ -lactam antibiotic groups in these strains strongly limits therapeutic options.

In this study, we report on the first detection of KPC-producing *K. pneumoniae* isolates in Hungary and their molecular epidemiology compared to the closely related ST11 isolates, described as the Hungarian epidemic clone III (ECIII) [8].

## Materials and methods

Between October 2008 and April 2009, nine *K. pneumoniae* isolates showing non-susceptibility to carbapenems were recovered from seven inpatients attending three hospitals in the north-eastern region of Hungary. The strains were submitted to the National Center for Epidemiology for confirmation of resistance mechanism and epidemiological typing. The nine isolates were obtained from the following samples: wound ( $n=2$ ), lower respiratory tract ( $n=2$ ), upper respiratory tract ( $n=2$ ), stool ( $n=2$ ) and central venous catheter ( $n=1$ ). Identification of the isolates was carried out by the Micronaut-E system (Genzyme Virotech GmbH, Ruesselsheim, Germany). The minimum inhibitory concentrations (MICs) of antibiotics for all isolates were determined by Etest (bioMérieux, Marcy l'Etoile, France) following the manufacturer's instructions and interpreted according to clinical breakpoints from the European Committee on Antimicrobial Susceptibility Testing (EUCAST, [http://www.eucastrg.org/clinical\\_breakpoints](http://www.eucastrg.org/clinical_breakpoints)). The putative production of a carbapenemase was tested by the modified Hodge test, and disk tests containing imipenem, meropenem or ertapenem either alone or combined with different carbapenemase inhibitors (750  $\mu$ g ethylenediaminetetraacetic acid, 835  $\mu$ g dipicolinic acid or 300  $\mu$ g 3-amino-phenyl-boronic acid) [9–12].

**Table 1** Clinical and in vitro characterisation of extensively drug-resistant (XDR) KPC-2-producing *Klebsiella pneumoniae* isolates

Isolate	Patient abbreviation	Sex/age <sup>a</sup>	Place of isolation <sup>b</sup>	Specimen	MIC (mg/L) <sup>c</sup>													
					CAZ	CTX	ETP	IPM	MEM	TET	TGC	CIP	GEN	AMK	SXT	CST		
K509/08	A	M/57	BCH	Upper respiratory tract	>256	256	8	16	8	16	2	>32	4	64	>32	0.5		
K97/09	F	F/67	MHC-SUH	Wound	>256	256	8	32	32	64	2	>32	4	64	0.5	16		
K105/09	C	F/62	BCH	Stool	>256	256	16	>32	16	32	4	>32	4	64	0.25	16		
K120/09	B	F/27	BCH	Upper respiratory tract	>256	32	16	>32	8	32	2	>32	2	64	0.5	32		
K132/09	G	M/85	BCH	Lower respiratory tract	>256	128	8	>32	32	16	2	>32	4	64	0.5	16		
K153/09	C	F/62	BCH	Wound	>256	256	32	>32	8	16	2	>32	2	64	0.5	16		
K160/09	D	M/66	MCH-D	Central venous catheter	>256	256	8	>32	32	16	2	>32	4	64	0.5	32		
K167/09	E	M/81	MCH-D	Lower respiratory tract	>256	64	16	>32	8	16	2	>32	2	64	0.5	16		
K168/09	B	F/27	BCH	Stool	>256	64	16	>32	>32	16	2	>32	2	64	0.5	32		

<sup>a</sup>M, male; F, female

<sup>b</sup>BCH, Borsod-Abaúj-Zemplén County Hospital, Miskolc; MHC-SUH, Miskolc Healthcare Center—Simmelweis University Hospital, Miskolc; MHC-D, Miskolc Healthcare Center-Diósgyőr, Miskolc

<sup>c</sup>CAZ, ceftazidime; CTX, cefotaxime; ETP, ertapenem; IPM, imipenem; MEM, meropenem; TET, tetracycline; TGC, tigecycline; CIP, ciprofloxacin; GEN, gentamicin; AMK, amikacin; SXT, trimethoprim/sulfamethoxazole; CST, colistin

The *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> genes were detected and characterised as described previously [8]. The presence of *bla*<sub>KPC</sub> genes in all isolates was verified using polymerase chain reactions (PCRs) followed by sequencing of all isolates for the determination of the KPC variant [13].

In order to separate the *bla*<sub>SHV-11</sub> from *bla*<sub>SHV-12</sub> variant, *bla*<sub>SHV</sub> PCR amplicons were digested with *NheI* (New England Biolabs, Ipswich, MA, USA) restriction endonuclease, which detects the G→A nucleotide gene substitution responsible for the glycine→serine substitution at codon 238. This mutation can be found only in SHV-type extended-spectrum beta lactamase (ESBL) variants, e.g. *bla*<sub>SHV-12</sub>. The *NheI*-undigested band was excised from an agarose gel, extracted using a Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA) and directly sequenced. Pulsed-field gel electrophoresis (PFGE) was performed according to the standardised CDC protocol on all Hungarian CTX-M-15-producing ST11 (allelic profile: 1-3-1-1-1-3-4) *K. pneumoniae* isolates (*n*=33), nine KPC-producing Hungarian and two KPC-2-producing Norwegian isolates (K47-25 and K48-58 [4]) as previously described [8].

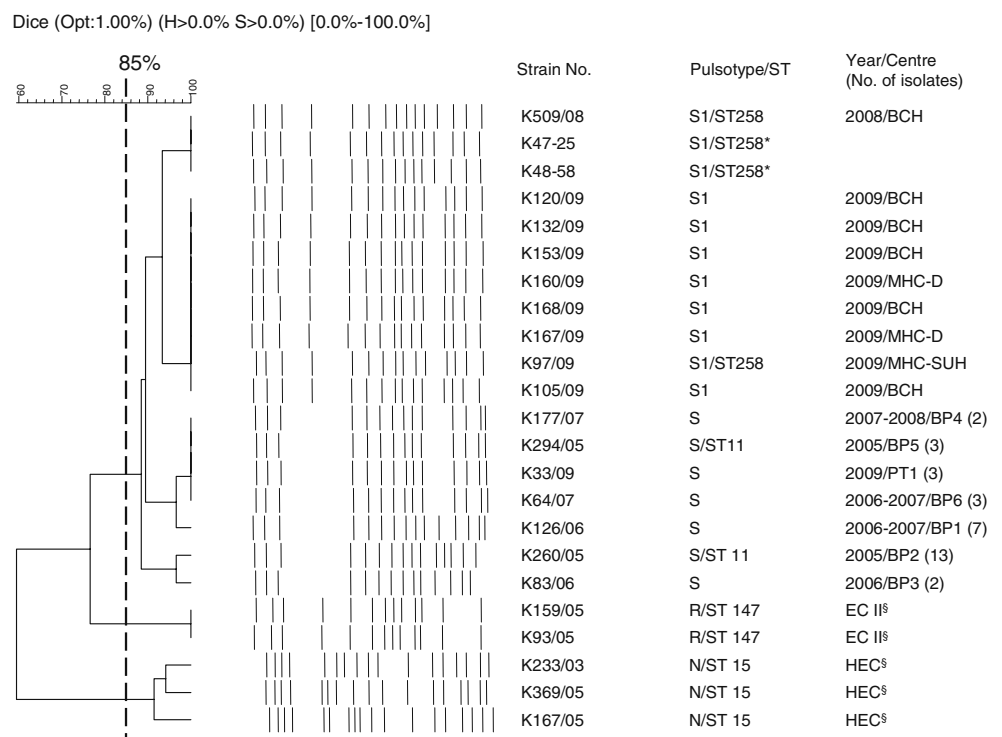
Multilocus sequence typing (MLST) with seven house-keeping genes was performed on two selected isolates according to Diancourt et al. [14]. Allele sequences and

sequence types (STs) were verified at the <http://www.pasteur.fr/mlst> web site.

### Results and discussion

The first KPC-producing isolate (K509/08) was recovered from a Hungarian patient (patient A) after direct transfer from a Greek hospital to the Borsod-Abaúj-Zemplén County Hospital in October 2008. Patient A was initially treated on the Neurosurgery ward and then in the intensive care unit (ICU) of the same hospital. From patient B who was treated at same time on the same ICU ward, isolate K120/09 was recovered in the beginning of 2009. The third patient (patient C) carrying isolates K105/09 and K153/09, as well as the fourth patient (patient D) carrying isolate K160/09, were treated on the Neurosurgery ward, similar to the patient A, but one month later. Then, patient D was transferred to the MHC-Diósgyőr in the same city and was treated on three different wards. Patient D and patient E carrying isolate K167/09 were treated in the ICU at the MHC-Diósgyőr simultaneously. KPC-producing isolates K97/09 and K132/09 were derived from two patients (patients F and G, respectively) with no history of hospitalisation together with patients A, B, C, D or E.

**Fig. 1** Cluster analysis of macrorestriction profiles (MRP) of nine KPC-2-producing and 33 CTX-M-15-producing *Klebsiella pneumoniae* strains from ten centres and sequence types (STs) of selected isolates. MRPs of strains representing ECII and HEC were added also. \*KPC-2-producing *K. pneumoniae* strains isolated in Norway [4]. §ECII, epidemic clone II; HEC, Hungarian epidemic clone [8]



\* KPC-2-producing *K. pneumoniae* strains isolated in Norway [4]

§ ECII, Epidemic clone II; HEC, Hungarian Epidemic clone [8]

The last KPC-producing isolate (K168/09) was recovered from patient B in April 2009. All patients suffered from critical illnesses and had a crude mortality of 100%.

All nine isolates showed extensively drug-resistant (XDR) phenotype, defined as resistance to all but one or two antibiotic classes [15]. The isolates were high-grade-resistant to extended-spectrum cephalosporins and ciprofloxacin, and resistant to ertapenem, imipenem and amikacin. In contrast, variable meropenem MIC values were detected (MIC range 8 to >32 mg/L). Eight isolates were intermediate susceptible to tigecycline, while isolate K105/09 was resistant (MIC 4 mg/L). In all isolates, elevated gentamicin MIC values were found. The MIC values of these antibiotics were very similar to the Norwegian and Swedish KPC-producing isolates [4].

Interestingly, the first isolate, K509/08, was highly resistant to trimethoprim-sulfamethoxazole (MIC >32 mg/L) and susceptible to colistin (MIC 0.5 mg/L), whereas the remaining eight isolates were susceptible to trimethoprim-sulfamethoxazole (MIC 0.25–0.5 mg/L) and highly resistant to colistin (MIC 16–32 mg/L) (Table 1). The majority of KPC-producing ST258 isolates reported from different countries and continents were susceptible to colistin, which is now a re-emerging antibiotic for the treatment of critically ill patients infected by multidrug-resistant (MDR) Gram-negative bacteria [16]. The emergence of colistin-resistant MDR *K. pneumoniae* was recently reported by Antoniadou et al. and the authors hypothesised that acquired resistance to colistin in MDR *K. pneumoniae* was due to selection pressure from excessive and inadequate colistin use [17]. However, none of the Hungarian patients carrying a KPC-producing strain were treated with colistin and it was not used in the given hospitals in this period; regardless of this, the resistance to colistin evolved very quickly. The isolates were negative for *bla*<sub>CTX-M</sub>, but all of them carried *bla*<sub>KPC</sub>, *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub>. Sequencing revealed that all isolates harboured *bla*<sub>KPC-2</sub> and the selected isolates (K509/08, K97/09) belonged to ST258 (allelic profile: 1-3-1-1-1-3-79) and harboured *bla*<sub>TEM-1</sub>, *bla*<sub>SHV-11</sub> and *bla*<sub>SHV-12</sub> also. Haeggman et al. [18] have shown that the class A chromosomal  $\beta$ -lactamase gene in *K. pneumoniae* evolve in parallel with housekeeping genes for at least 6 million years. We suppose that the chromosomally encoded SHV variants might be characteristic of *K. pneumoniae* clones. In this way, the presence of chromosomally encoded non-ESBL *bla*<sub>SHV-11</sub> [19] in both ST11 and ST258 (a single-locus variant of ST11) isolates is in agreement with their possible clonal relationship.

PFGE analysis of KPC-producing Hungarian and Norwegian ST258 isolates, as well as of all the exclusively CTX-M-15-type ESBL-producing Hungarian ST11 isolates (ECIII, pulsotype S, 2005–2009), revealed the existence of one genetic cluster at an 88% similarity level, which could

be subdivided into two main subclusters (Fig. 1). The ST11 (ECIII) showed quite a homogeneous structure during nearly five years of nationwide dissemination after its first detection in 2005. The same genomic stability was found in all KPC-producing ST258 isolates: the two Norwegian isolates and the first Hungarian KPC-producing *K. pneumoniae* isolate showed indistinguishable PFGE patterns, despite a one-year difference in time and place of isolation.

According to the MLST database, ST11 was identified not only in Hungary, but in France in 1997, the Netherlands, Spain and Korea (<http://www.pasteur.fr/mlst>). The single-locus variant of ST11, ST258, has been recently found in Norway, Sweden [4], Israel, the USA [3], Poland [5], Finland [6], Italy [7] and Korea (<http://www.pasteur.fr/mlst>). Considering that the clonal complexes (CCs) were defined as sequence types with allelic difference in no more than one locus from at least one other profile of the group [20] and according to the PFGE clustering, MLST results and eBURST analysis [21] and the occurrence of SHV-11 in both ST11 and ST258, it seems that this is the first hyperepidemic clonal complex of MDR *K. pneumoniae* which encompass ST11, ST258, ST270, ST340, ST379, ST407 and ST418, possibly undergoing worldwide spread. Both ST258 and ST340 stand out as likely founders of this CC (CC258/CC340), and further sampling will be necessary to understand the diversity and evolution of this CC.

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